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New approaches to cancer vaccination and immunotherapy, based on the optimization of the presentation of tumor antigens and the stimulation of CD4 positive T Helper cells.

Thesis of

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"Un viaggio di mille miglia comincia sempre
con il primo passo"

Lao Tzu

Ai miei genitori ed alle persone a me care,
perché il loro bene mi ha sempre sostenuta.

ABSTRACT AND GOALS OF THE THESIS

Recently we have shown that a number of murine tumors arising from mice of the Balb/c (MHC H-2d) strain, induced to express MHC class II molecule after transfection with CIITA (MHC class II transactivator) *(Accolla R.S. et al. 1986)* can be rejected effectively, generating tumor specific T helper cell (TH) triggering, immunological memory and protection even against parental tumor (*Meazza R. et al. 2003 ; Frangione V. et al. 2010; Mortara L. et al. 2006)* . The aims of my PhD thesis were: a) - to assess whether the same results can be extended to tumors of different MHC background (H-2b); b) - to investigate, using knock-out C57Bl mice for dendritic cells, whether CIITA transfected tumor cells can act as "surrogate APC" for their tumor antigens *in vivo*. To this end, LLC (Lewis lung carcinoma, H-2b) cells were stably transfected with CIITA and selected for expression of MHC class II molecules. Parental tumor cells and CIITA-transfected cells (LLC-CIITA) were injected subcutaneously into C57/BL6 mice and tumor take and growth kinetics were assessed. Mice injected with LLC-CIITA cells were tumor-free for longer time than mice injected with parental tumor cells. The growth kinetics and the size of CIITA-expressing tumors were significantly reduced compared to the parental tumor. Adoptive cell transfer of purified CD4+ TH cells from mice injected with LLC-CIITA into naïve mice demonstrated that these cells were able to protect from LLC parental tumor growth. Taken together these results strongly suggest that, similarly to H-2d strain, also H-2b tumors can be rejected if expressing CIITA-mediated MHC class II molecules, confirming the general applicability of our tumor vaccination model. To achieve the second goal we performed *in vivo* experiments in a novel transgenic mouse model named CD11.c DOG, in which it is possible to induce a conditional depletion of dendritic cells (DC) by diphtheria toxin (DT) treatment. These mice express the diphtheria toxin

receptor (DTR) under the control of the promoter of the CD11c molecule, expressed preferentially in DC. Once injected with DT, dendritic cells are eliminated for the period of treatment up to 12 days. DT-treated CD11c.DOG mice where injected with LLC-CIITA tumor cells and tumor take and tumor growth was followed during time. We found that LLC-CIITA cells can be recognized and rejected better than parental tumor even in CD11c-DOG mice. These results suggest that CIITA-tumor cells may act in vivo as surrogate APCs for their own tumor antigens and trigger and adaptive immune response mediated by CD4+ TH cells.

ABBREVIATIONS

ACT	Adoptive Cell Transfer
APC	Antigen Presenting Cell
CIITA	Class II Transactivator
CTL	Cytotoxic T Cell
DC	Dendritic Cell
DT	Diphteria Toxin
DTR	Diphteria Toxin Receptor
HLA	Human Leukocyte Antigens
IFN-γ	Interferon- γ
li	Invariant chain
LLC	Lewis Lung carcinoma
MDSCs	Myeloid-derived suppressor cells
MHC	Major Hystocompatibility Complex
TAA	Tumor associated antigen
TH	T Helper cell
TIL	Tumor- Infiltrating Lymphocytes

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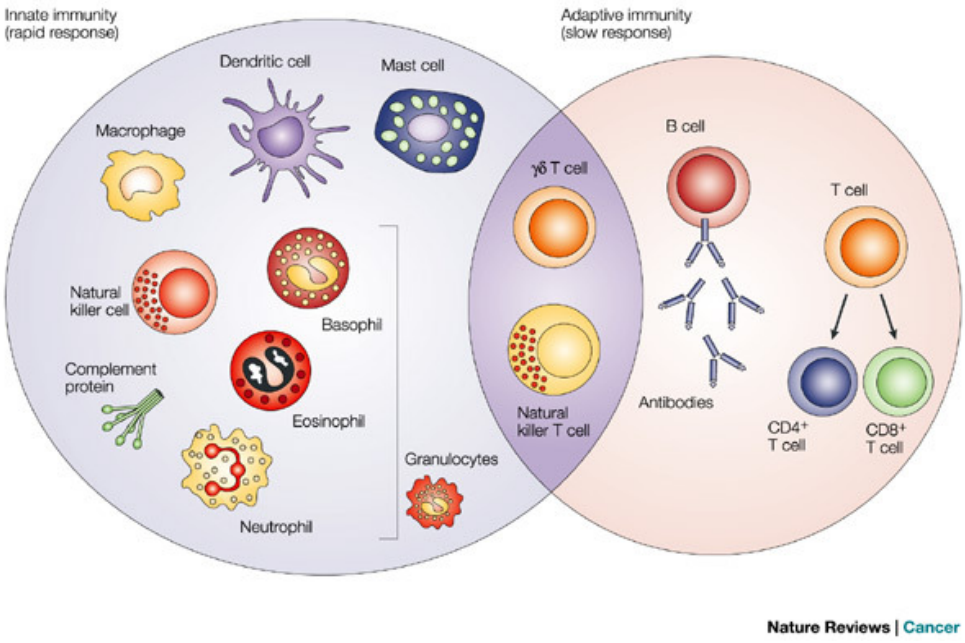
1 INTRODUCTION

1.1 The Innate and Adoptive Immunity

The immune system is a complex network of interacting cells, cell products, and cell-forming tissues that work together to protect the body against attacks by "foreign" invaders. The targets of the immune system are primarily organisms such as viruses bacteria, parasites. The immune system encompasses various types of cells including T and B lymphocytes capable of specifically recognize antigens via their clonotypically distributed receptors (adaptive immunity), as well as other hemopoietic derived cells such as macrophages, dendritic cells, granulocytes and NK cells (innate immunity). In the adaptive immunity, B cells are responsible of producing and secreting antibodies that circulate in the blood and bind to antigens on infectious agents as these molecules are primarily responsible for protection against bacteria and viruses. This interaction can result in direct inactivation of the microorganism or activation of a variety of inflammatory mediators that will destroy the pathogen ***(Fig. 1)***. T cells are a subset of lymphocytes that undergo differentiation during an immune response and develop into several subpopulations of effectors. We distinguish two major types of T cells. Helper T cells (TH) expressing the CD4 cell surface marker are the primary cells involved in the recognition of antigen presented by HLA class II molecules. These cells are needed for maturation and differentiation of all other cells of adaptive immunity. Cytolytic T cells (CTL) expressing the CD8 cell surface marker develop into cytolytic effectors (CTL, cytolytic T lymphocytes) that attack and kill target cells directly. Targets for CTL include cells infected by viruses, as well as cells that have become cancerous. This arm of adaptive immunity is termed *cell-mediated immunity*. To initiate an effective

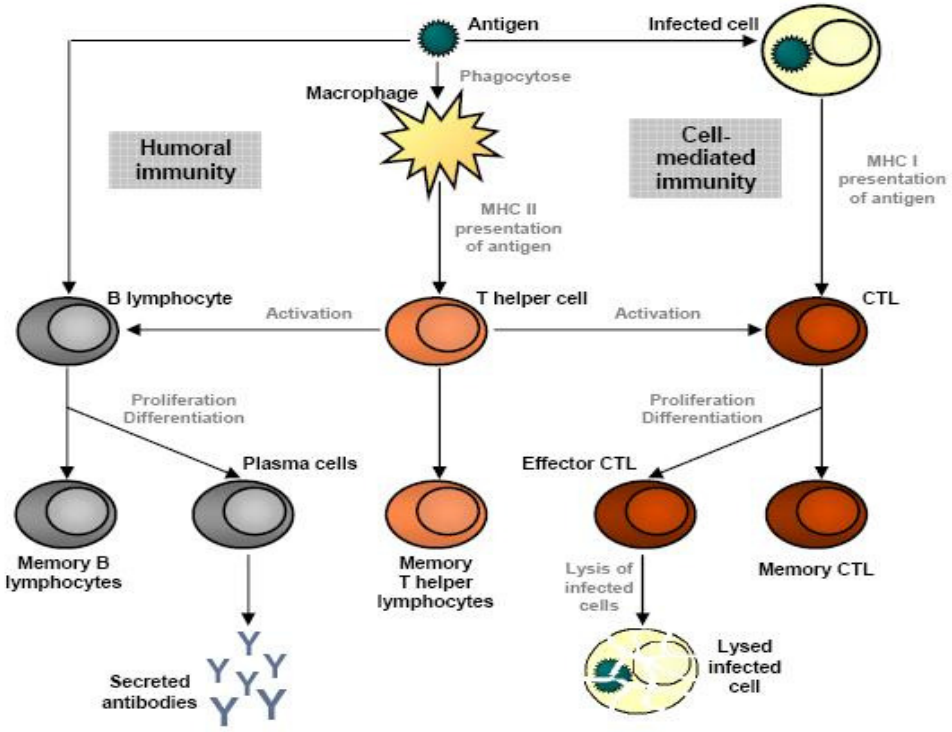
immune response, most antigens must be processed; because they cannot react directly with cells of the immune system, the antigens must be shown or presented to the immune cells in a specific manner. This is accomplished by antigen-processing and presenting cells (usually dendritic cells and macrophages), generally referred as APCs ***(Fig. 2)***. T cells develop in the thymus, and here they acquire their competence and specificity by recognizing self antigens presented via the MHC molecules expressed by the individual. CD4+ TH cells become restricted by MHC class II molecules and CD8+ T cells become restricted by MHC class I molecules. Cells recognizing cell constituents with high affinity are deleted in the thymus (central tolerance) and only self-restricted T cells with low affinity are matured and exported in peripheral lymphoid organs.



(From Glenn Dranoff, Nature Reviews Cancer 2004)

Figure 1. *Diagrammatic representation of Innate and Adaptive Immunity.*

The innate immune response functions as the first line of defence against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. The adaptive immune response is slower to develop, but manifests as increased antigenic specificity and memory. It consists of antibodies, B cells, and $CD4^+$ and $CD8^+$ T lymphocytes. Natural killer T cells and γδT cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity.



(From Janeway CA Jr, Travers P, Walport M, Shlomchik MJ, 2005.)

Figure 2 . *Humoral and cell-mediated immunity*

TH cells has a central role in immune response. TH cells recognize MHC II-restricted antigen on the surface of APC (e.g. macrophage) inducing the activation of CTL and B lymphocytes. CTL proliferate and differentiate into memory and/or effector CTL that kill infected and cancer cells. B cells proliferate and differentiate into memory B lymphocyte and/or plasma cells secreting antibodies.

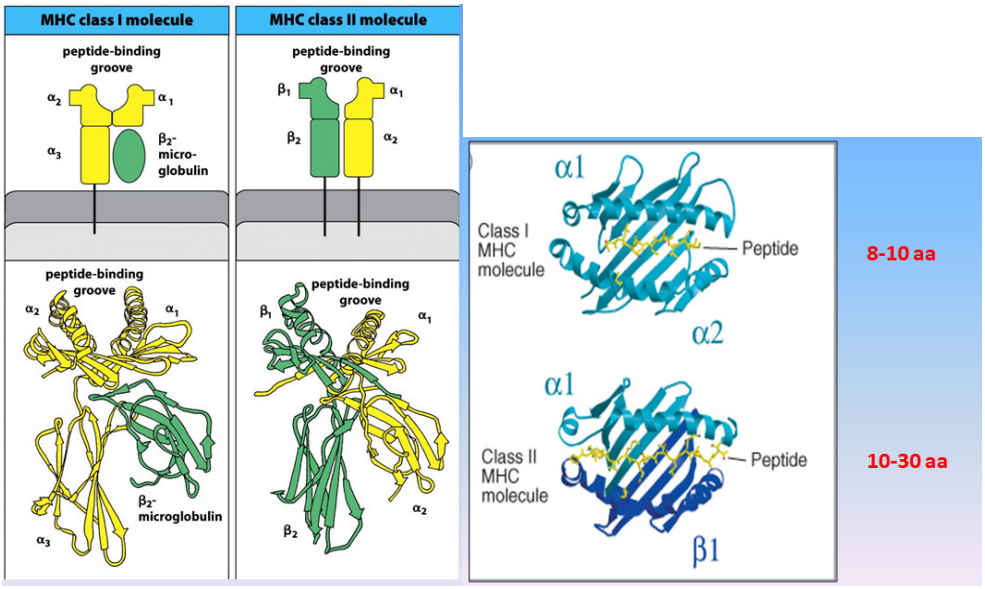
1.2 MHC and CIITA

Class I and class II MHC molecules share some structural features which are essential for the presentation of peptides and antigen recognition by T lymphocytes. Via their T cell receptor (TcR), CD4 T lymphocytes selectively recognize antigenic peptides bound to MHC class II molecules whereas CD8 T cells recognize antigenic peptides bound to MHC class I molecules. Class I molecules are formed by two non-covalently linked polypeptide chains: an MHC-encoded α chain (or heavy chain) of 44-47 kDa and a non-MHC encoded subunit, non transmembrane, non covalently bound to α-chain, of 12 kDa called β2-microglobulin (invariant but essential). Each α chain is oriented in such a way that about three quarters of polypeptide extend into the extracellular milieu; a short hydrophobic segment crosses the cell membrane and the terminal carboxylic acid is localized inside the cell. The N-terminal domains α1 and α2 present the polymorphic residues that contribute to the variability of class I alleles and form a platform consisting of a leaflet of β antiparallel strands, which support two parallel strips of α-helix. This set constitutes the pocket able to bind peptides of 8-11 amino acids in a flexible extended conformation; the pocket ends are closed in such a way that do not accommodate large peptides. This pocket will interact with the TcR expressed on the surface of CTL. The α3 domain of the α chain is conserved in all class I molecules. This segment contains the binding site for the CD8 molecule expressed on CTL. Class II MHC molecules are composed by two non-covalently associated polypeptide chains, an α chain and a β chain. Unlike class I molecules, the genes that code for both chains are polymorphic. The amino terminal segments α1 and β1 form the peptide binding site. The ends of the pocket are open; this implies that class II molecules bind peptides larger (10-30 aa) than those of class I. α2 and β2

segments of the class II molecules, as the segment α3 and β2-microglobulin of MHC class I, are folded in Ig domains that do not vary substantially between the different alleles. The basic characteristics of both MHC class I and class II molecules are summarized in ***Figure 3***. The gene encoding MHC molecules are located in human chromosome 6 (the HLA system) and in murine chromosome 17 (H-2 system) (see ***Table 1*** and ***Figure 4***). Polymorphic human class I molecules are encoded by at least three loci designated : HLA-A, HLA-B, HLA-C whereas class II molecules are encoded by six loci divided in three subloci: HLA-DR, HLA-DQ, HLA-DP. The mouse MHC region, designated H-2, is organized similarly to human MHC region. Mouse class I equivalents include H-2K,-D and-L, whereas class II is made up of at least four loci organized in two subregions, A and E subregions. H-2d and H-2b haplotypes have different numbers of class I and class II genes. Most interestingly, mice of the H-2b haplotype have class I K and D products but they miss L products. Furthermore H-2b haplotypes do not express the MHC class II molecule of the E locus because they do not express the EA gene encoding the alpha chain. While MHC class I gene products are expressed in virtually all cells, MHC class II gene products are expressed in a limited set of cells, and particularly in cells that serve as antigen presenting cells for CD4+ TH cells. Many other genes, non polymorphic in nature, are located within the MHC system of both mouse and human, and interestingly their products are mostly correlated with the function of the immune system. Thanks to their location these genes can be regulated in a coordinated manner. TAPBP gene codes for tapasin, a chaperone protein; LMP gene codes for LMP2 and LMP7, two proteosome subunits, and TAP gene code for proteins Transporters associated with Antigen processing. All these proteins are essential for a normal antigen presentation and MHC- I expression. The expression patterns of MHC class I and MHC class II molecules and their cell biology reflect their different roles (***Table 1 and Fig. 4***). MHC class I molecule

present endogenously synthesized antigens, e.g. viral proteins to CD8+ T cells. MHC class II molecules present exogenously derived proteins, e.g. bacterial products or viral proteins from phagocytosed viruses to CD4+ T cells. Expression of MHC class II molecules is exquisitely controlled at the transcriptional level and a large set of proteins interact with promoters of class II genes. The most important is the class II transactivator (CIITA), a master controller that orchestrates expression but does not bind directly to the promoter. *(Ting et al., 2002).* The MHC II transactivator was identified and characterized by a somatic cell approach. After generation of a somatic mutant (RJ 2.2.5), negative for MHC class II expression *(Accolla R.S. et al., 1983)*, somatic complementation with murine MHC class II positive cells demonstrated the existence of a dominant locus, encoding the *trans*-acting activator. This locus was mapped to mouse chromosome 16 and designated *Air-1 (Accolla R.S.et al. , 1985; Accolla R.S et al. , 1986)* and the corresponding human locus designated *AIR-1*. Seven years later, the product of the *AIR-1* locus was cloned by a gene complementation approach and named CIITA *(Steimle et al., 1993).* It is now well established that transcription of the *AIR-1* gene is regulated by multiple promoters. Four promoters – referred to as pI, pII, pIII and pIV - have been defined. Each of these promoters is responsible for distinct tissue specific expression modes of CIITA. Promoters pI and pIII are responsible for myeloid and lymphoid specific expression patterns, respectively. A specific role for pII is not currently known. Promoter pIV is the major IFN-γ responsive promoter functioning in non-hematopoietic cells *(Reith and Boss, 2008)* (***Fig. 5***) . Unlike other protein factors that directly bind DNA and have ubiquitous expression, CIITA has tissue-specific expression and is always associated with the expression of class II genes *(Harton JA, 2000).* CIITA protein consists of an acidic transcriptional activation domain, a phosphorylation and dimerization domain (PS/T*) (Tosi et al. Embo Journal, 2002),* a GTP binding domain and a 4

LRRs (leucine-rich repeats) (***Fig. 6***). The protein is located mostly in the nucleus and acts as a positive regulator of class II major histocompatibility complex gene transcription. By binding GTP, CIITA facilitates its own transport into the nucleus. Once in the nucleus it does not bind DNA. In fact CIITA integrates the function of several DNA-binding proteins with specific characteristics. By interacting with the RFX protein complex (RFX5, RFXANK, RFXAP), the NF-Y complex (NF-YA, B, C) and CREB, it facilitates the assembly and the binding of these complexes to class II promoter. By interacting with the histone acetyl transferases CBP and pCAF, it facilitates the remodeling of chromatin around the promoter. By interacting with TAFs and with the P-TEFb (Cyclin T1-CDK9) complex, CIITA facilitates the initiation and the elongation of transcripts, respectively (see ***Fig. 5***).



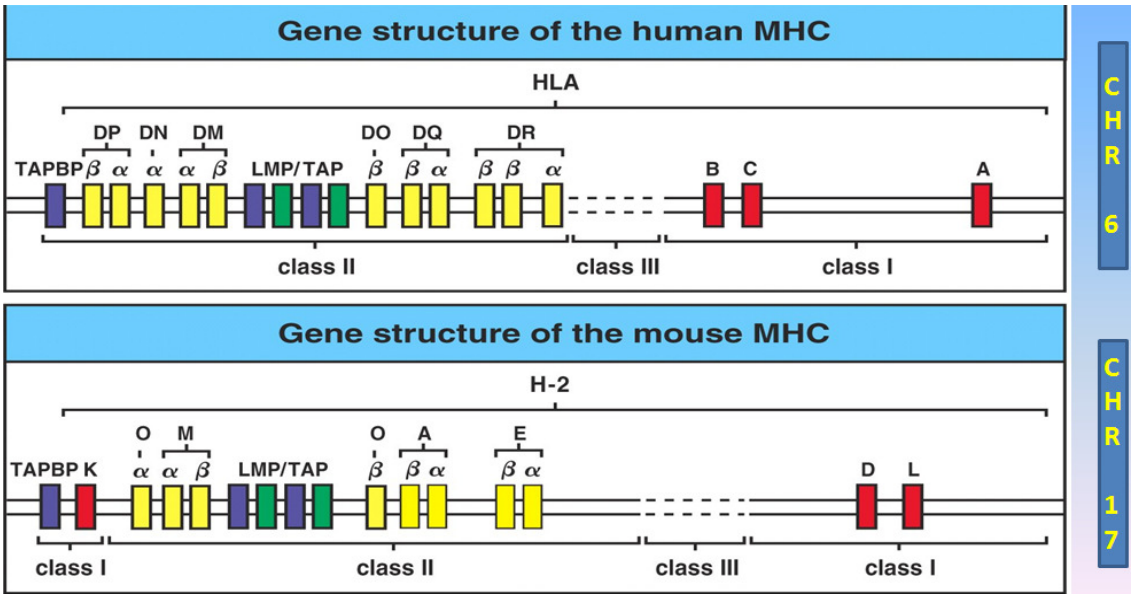
(Modified from A.K. Abbas, A.H. Lichtman, 2003)

Figure 3. *Structures of Histocompatibility molecules and of peptide binding to MHC molecules.*

Tissue/cell	MHC	
	class I	class II
Hematopoietic		
T cells	+++	+*
B cells	+++	+++
Macrophages	+++	++
Dendritic cells	+++	+++
Neutrophils	+++	—
Erythrocytes	—	—
Non-hematopoietic		
Thymic epithelium	+	+++
Liver hepatocytes	+	—
Kidney epithelium	+	—
Brain	+	— †

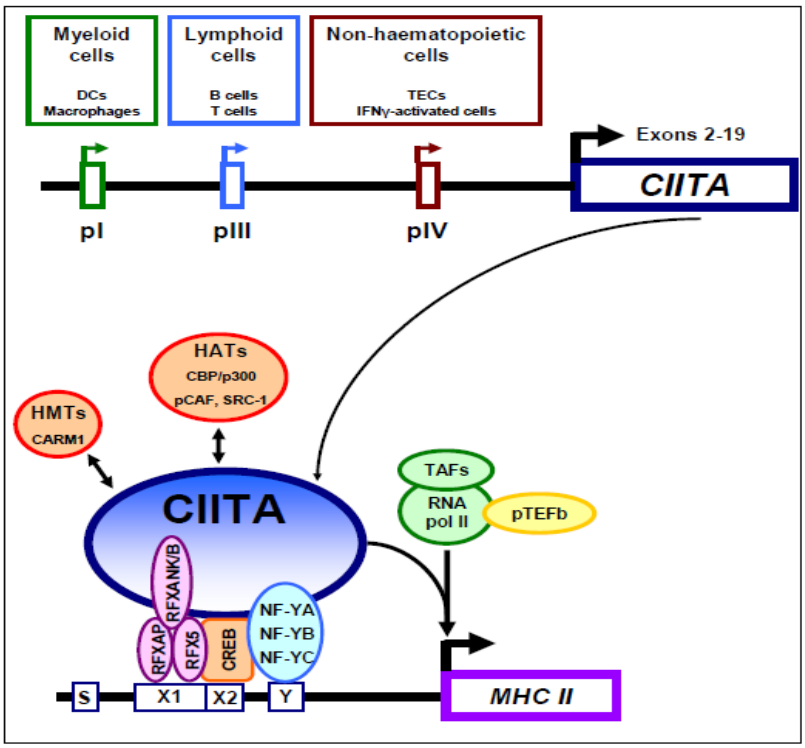
(From A.K. Abbas, A.H. Lichtman, 2003)

Table 1. *Expression and distribution of MHC molecules in different cell types*



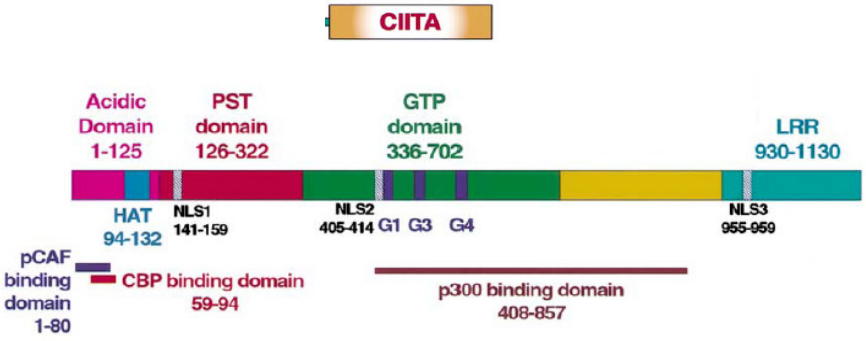
(Modified from A.K. Abbas, A.H. Lichtman, 2003)

Figure 4. *Schematic representation of Human (HLA) and Mouse (H-2) MHC regions.*



(From Reith W. and Boss J M., 2008)

Figure 5. *The promoter of MCH class II gene. For further explanation see the text.*



(From Ting JP. and Trowsdale J. , 2002)

Figure 6. *The structure of CIITA protein. For further explanation see the text.*

2. ANTI-TUMOR IMMUNITY

2.1 Causes of scarce and insufficient response against the Tumors

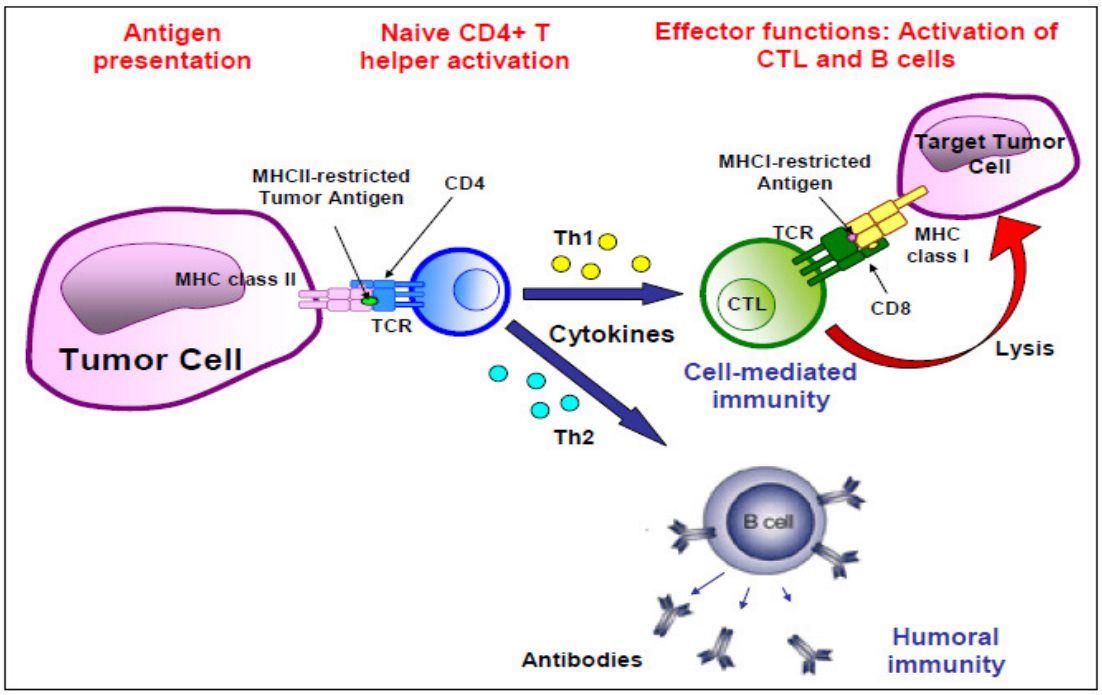
Although the host can mount an immune response against cancer cells, the fact that the tumor takes off in cancer patients demonstrates that tumor may elude immune defences *(Vesely M. et al., 2011; Rosenberg SA. et al., 2004)* .Tumors are complex tissues. They are composed of tumor cells, tumor stroma and in many cases of a series of blood-derived infiltrating leukocytes including cells of innate and adaptive immunity. It has been found that tumor-infiltrating leukocytes, including neutrophils, macrophages, mast cells, eosinophils (components of an heterogenous family of myeloid cells which can acquire inhibitory activity on immune lymphocytes, the so-called Myeloid-Derived Suppressor cells (MDSC) *(Gabrilovich DI . et al. 2012)* as well as T cells with CD4+/CD25+ phenotype and suppressive function on helper and effector T cells, designated regulatory T cells (Tregs) *(Sakaguchi S. et al. 1995; Nishikawa H. and Sakaguchi S., 2010),* may cooperate in favouring, instead of antagonizing, tumor growth. These findings have created a diffuse belief that pro-tumor polarization of the innate and adaptive immunity is the cause for tumor cells to survive, replicate and spread *(Mantovani A. and Sica A. , 2010; Ruffell B. et al. 2009).* With the final goal to generate a stronger and sustained adaptive immune response against the tumor that may overcome the intrinsic difficulties of poor immunogenicity of TAA and the ostile tumor microenvironment, and assuming that the most potent anti-tumor effector is the CTL, many investigators have purified CTL-defined antigens, particularly from tumor tissues and used them as vaccine. However in most studies the CTL

responses were weak and unable to control tumor growth and metastasis *(Rosenberg SA et al.,2008).* Beside the reasons describe above, additional important elements did come into play. For example the frequent loss or reduced expression of MHC-I molecules in tumor cells which prevents TAA presentation to CTL *(Kageshita T. et al. 1999, Garrido F. et al. 2010; Ossendorp F. et al.1998)*, and importantly the poor tumor-specific, MHC-II-restricted T cell help generated in tumor-bearing patients. The latter element is of fundamental importance because TH cells are required for optimal induction of both humoral and cellular effector mechanisms and particularly for CTL maturation, clonal expansions and acquisition of cytolytic function. TH cell triggering requires recognition of antigenic peptides presented by MHC-II molecule expressed on professional antigen presenting cells (APC) including dendritic cells (DC), macrophages and B cells. Preventing and/or inhibiting the phase of MHC-II-dependent tumor antigen presentation to and/or activation of TH cells would thus be an effective strategy to block the adaptive anti-tumor immune response from its beginning.

3 OUR APPROACH

3.1 Previous Results

The approach followed by our laboratory to obtain an optimal TH anti-tumor response is based on the concept that if MHC-II molecules can be expressed in tumor cells (most tumors do not express MHC class II) these cells may function as surrogate APC for their own tumor antigens and thus trigger an effective immune response. To this direction, cells were engineered with the cDNA coding for CIITA. Indeed we demonstrated that CIITA-induce MHC class II positive tumor cells can be recognized in vivo and elicit both tumor-specific CTL and, more importantly, CD4+ TH cells (*Meazza et al., 2003; Mortara L. et al., 2006; Mortara L. et al., 2009; Frangione V. et al., 2010;)*. This approach has shown promising results in a series of tumor cell lines from mice of Balb/c strain (H-2d). CIITA transfected tumors of distinct histological origin (WEHI fibrosarcoma, C51 colon adenocarcinoma, RENCA renal adenocarcinoma, TS/A mammary adenocarcinoma) can be rejected effectively generating tumor specific T helper cell (TH) triggering, immunological memory and protection even against parental tumor (***Fig. 7***).



(From V. Frangione, PhD Thesis at the University of Insubria, 2008)

Figure 7. *Anti -tumor Adaptative Immune Response.*

Tumor cell stably transfected with CIITA and expressing MHC II molecules is depicted here as a possible antigen-presenting cells (APCs), presenting MHC II-restricted tumor antigen. Helper T cells recognize these tumor antigens, with the help of CD4 co-receptor (CD4+) expression. The activation of a naïve helper T cell causes it to release cytokines and other stimulatory signals that determinate the activation of CTL that lyse target tumor cell (established) and B cells that produce antibodies (not yet proven).

3.2 Present Thesis Approach

The main purposes of my thesis where two fold. First to validate the approach of *in vivo* immunogenicity and generation of a protective anti-tumor immune response with CIITA-tumor cells in a mouse strain of different genetic background. Second, to define more stringent methodologies for assessing the real APC function *in vivo* of CIITA-tumor cells and particularly the possibility that these cells may indeed prime naïve CD4+ TH cells to become anti-tumor specific T cells without the participation of classical professional APCs. As tumor model system we used the Lewis Lung Carcinoma (LLC) of C57BL/6 mouse origin (H-2b). LLC cells were stably transfected with the cDNA coding for CIITA and used as prophylactic cancer vaccine. The results presented here demonstrate that a significant percentage of mice injected with LLC-CIITA cells rejected the tumor and an high percentage remained tumor-free for a longer time than mice injected with parental tumor cells. Furthermore, the growth kinetics and the size of CIITA-expressing tumors are significantly reduced compared to the parental tumor. CD4+ and CD8+ lymphocytes isolated from spleen of mice rejecting the CIITA-tumor were able to protect or strongly retard the growth of parental LLC tumor cells in naïve recipients, demonstrating the importance of inducing a TH cell response for the generation of adaptive anti-tumor immunity. Furthermore, the use of a transgenic H-2b mouse model in which a conditioned depletion of professional APCs (dendritic cells) can be generated, demonstrated that CIITA-tumor cells could still be rejected *in vivo* because of specific immunity, strongly suggesting that these cells act as surrogate APC for priming and induction of anti-tumor TH cells.

4. MATERIALS AND METHODS

4.1 Cell culture and in vitro LLC cells growth kinetics

The murine tumor cell line LLC (Lewis Lung Carcinoma) was cultured in DMEM medium supplemented with L-glutamine and 10% of heat-inactivated FBS in a 5% CO2 atmosphere at 37^0C. The *in vitro* growth kinetics in absence and in presence of G418 (the selective neomycin antibiotic for growing CIITA transfectants) was assessed by plating the cells at fixed numbers and by counting them at fixed times. The dose of G418 to be used for selecting the transfectants was assessed in similar way.

4.2 Short-term treatment of parental LLC cells with IFN-γ and evaluation of surface MHC-I and MHC-II molecules expression

In order to assess the steady state and the inducible (IFN-γ) MHC class I and class II expression in LLC parental cells (LLC pc), the cells were plated at different cell number in each well of a six well plate. After 24 hours 300 U/ml of IFN-γ was added to each well. After 48h, 72h and 96h of IFN-γ treatment, the cells were tested for MHC-I and MHC-II cell surface molecules expression by immunofluorescence and cytofluorometry in a Fluorescence activated cell sorter (FACS) Becton-Dickinson Aria.II instrument using B22.249 biotinalated (anti-H-2Db) and AF6120.1 (anti-H-2 A^b) antibodies and as a second step reagent a FITC Streptavidin (Biolegend, Mylan , Italy) antibody.

4.3 Plasmids, CIITA transfection and cell surface phenotyping

The full-length human CIITA cDNA was obtained by preparative proof-reading PCR (Fusion Taq; Finnzymes, Helsinki, Finland) from pREP10-CIITA plasmid, *(Sartoris et al. 1996).* Forward and reverse primers bear *Xho-I* linker tails used to subclone the PCR product into the *pLXIN2.ape* retroviral vector (obtained from Dr. Antonio Daga, Department of Translational Oncology, IST-National Cancer Research Institute, Genoa, Italy) , modified from pLXIN retroviral vector (Clontech, Milan, Italy) to include additional restriction sites in the multiple cloning sequence. The resulting pLXIN-CIITA vector was transfected by using Lipofectamine ™ 2000 (Invitrogen SRL, San Giuliano Milanese, Italy), a molecule composed by a cationic tail (to which it binds the DNA) and a lipid region (which facilitates the DNA entry across the plasma membrane). Briefly, parental LLC tumor cells were transfected with 2 μg of plasmid or with pLXIN empty vector as a control. The plasmid carries a gene conferring resistence to neomycin. After transfection, the cells were cultured in DMEM medium supplemented with 1mg/ml of neomycin (G418, Sigma, Milan, Italy). Both trasfectants were tested for MHC-II surface expression by cytofluorimetry using anti-mouse I-A^b antibody AF6120.1 To obtain LLC transfectants with a stable and high expression of MHC-II molecules induced by CIITA, cells were sorted several times and subsequently cloned by limiting dilution procedures. Most of the studies described in this work were performed by using the LLC-CIITA transfectant clone E10.

4.4 Murine Models

Five to eight week-old C57BL/6 (H-2K^b) mice were purchased from Charles River - Research Models and Services (Calco, Italy). CD11c-DOG transgenic mice were kindly provided by Dr Günter J. Hämmerling (German Cancer Research Center DKFZ Heidelberg). CD11.c-DOG transgenic mice were generated by gene targeting using a fusion construct composed of the DNAs for the human DTR, ovalbumin fragment aa 140–386, and eGFP inserted at the start codon of the CD11c gene The eGFP component of the fusion protein failed to give a fluorescence signal, whereas the ovalbumin protein was expressed in dentritic cells (DC) (***Fig.17***). CD11c-DOG heterozygous knockout mice were propagated by crossing heterozygous knockout males to C57BL/6 females. Mice were held under specific-pathogen-free conditions at the animal facility of the University of Insubria, and experiments were performed according to institutional guidelines and regulations. Genomic DNA was isolated from mouse tails using SNET solution (Tris-HCl pH 8.0 1.0M, EDTA pH 8.0, NaCl 5 M, SDS 10%) Proteinase K 20mmg/ml (Sigma, Mylan, Italy) for 3 hrs at 50 ^{o}C and subsequently, using phenol chloroform extraction method. Genotyping was carried out by PCR from genomic DNA of tail biopsies using the following primers for OVA:

5'-AACCTGTGCAGATGATGTACCA-3'(*sense*) and

5'-GCGATGTGCTTGATACAGAAGA-3'(*antisense*). β–2 microgobulin was used as housekeeping gene and detected with the following primers:

ßm: 5'-CACCGGAGAATGGGAAGCCGAA-3'(*sense*) and

5'-TCCACACAGATGGAGCGTCCAG-3'(*antisense*).

Amplified DNA samples were analyzed by 1,5% agarose gel electrophoresis and photographed (***Fig. 18***).

4.5 In vivo injection of LLC parental and LLC-CIITA tumor cells and determination of their growth kinetics

Groups of 10 C57BL/6 female mice, aged five to six weeks, were injected subcutaneously in the right flank with 3 x10^5 LLC parental cells (LLCpc), LLC transfected with empty vector (LLC-Lxin), or transfected with CIITA (LLC-CIITA). As control, a group of 5 mice were injected using the same number of LLC cells transfected with the empty vector. Tumor size was measured using caliper at weekly intervals and was expressed as a multiple of the wider and smaller tumor diameters.

4.6 Ex vivo tumor cell culture and MHC-II cell surface phenotyping

To assess the stability of the MHC class II phenotype *in vivo* LLCpc and LLC-CIITA tumors were excised and collected in DMEM medium supplemented with penicillin and streptomycin in a 50 ml centrifuge tube and kept on ice prior to processing in a sterile tissue culture hood. Each tumor was mechanically dissociated using a 70 µm filter, sterile needle, scalpel and pestle. The cell suspension was collected in a tube and centrifuged. The cell pellet was resuspended in complete DMEM medium supplemented with specific antibiotics and cultured in a 5% CO2 atmosphere at 37^0C. Quickly after establishing the cell culture, expression of MHC-II cell surface molecules was analyzed as described above.

4.7 Conditional DC cells depletion in CD11c.DOG mice

For systemic DC depletion, CD11c.DOG mice were injected i.p. with 8ng/gr of body weigh (gbw) of diphtheria toxin (DT) (Sigma) dissolved in PBS. All experiments were performed on heterozygous CD11c.DOG mice of about 2 months of age. Wild-type C57BL/6 mice of the same litter and age were used as controls. Conditional DC depletion was tested in the spleen. After 48h of DT treatment, mouse spleens were harvested and collected in DMEM medium supplemented with penicillin and streptomycin, mechanically processed using a 40 μm filter, sterile needle and pestle. The cell pellet was collected by centrifugation, and ACK solution was added to lyse the erythrocytes. The supernatant was discarded and the pellet was then resuspended in DMEM medium. Splenocyte viability was measured using trypan blue. Splenocytes were phenotypically analyzed by direct immunofluorescence and FACS analysis.

4.8 Cell surface phenotype of mouse splenocytes

Spleen cells from normal or tumor bearing mice were assessed by FACS for the expression of several cell surface markers by using directly fluorochrome-labeled antibodies and corresponding isotype control antibodies. The relevant cell surface markers assessed were: CD11c, CD4, CD8 and CD19.

4.9 Adoptive Cell Transfer

In order to evaluate the ability of splenocytes from CIITA-tumor vaccinated mice to confer protection to naïve syngeneic recipients the spleen was isolated under sterile conditions, dissociated with a sterile syringe plunger in cell strainer with 40 μm nylon mesh (BD Milan, Italia), treated with ACK buffer (150 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA pH7.4) to deplete red blood cells. $CD4^+$ and $CD8^+$ cells were purified from the total spleen suspension using the $CD4^+$ or $CD8^+$ T Cell Isolation Kit II MACS (Miltenyi Biotec GmbH, Germany), according to the manufacturer' instruction. Purification was by positive selection of non-CD4 or non CD8-positive population and collection of the relevant flow-through CD4+ or CD8+ populations, respectively. Purity was ≥ 90%, as assessed by immunofluorescence and FACS analysis. Total splenocytes, purified $CD4^+$ or $CD8^+$ cells from either immunized or naïve mice were separately mixed with parental tumor cells in RPMI 1640. Groups of naïve C57BL/6 mice (5 to 8 weeks-old) were s.c. injected with $5x10^4$ parental tumor cells plus total splenocytes (ratio 1:20) or purified $CD4^+$ or $CD8^+$ cells maintaining the same proportion observed in the total spleen. Tumors were measured weekly by caliper and sizes recorded as the tumor area (mm^2).

4.10 Evaluation of $CD11b^+$ and $Gr1^+$cells

Several studies have shown the presence of splenomegaly and accumulation of $CD11b^+$ and $Gr1^+$ cells in the spleen of tumor-bearing mice. In order to investigate this aspect in our tumor vaccination model, splenocytes from LLC-CIITA tumor-free mice, as well as from LLCpc tumor-bearing mice were isolated from spleens. Specifically, each spleen was harvested under sterile conditions, and spleen cells prepared as described above. Single cell suspensions were incubated with anti-Gr1 or anti-CD11b specific antibodies and analyzed by FACS. To assess the function of CD11b+/Gr1+ cells two groups of five C57BL/6 mice were injected with $1x10^6$ FACS-sorted $CD11b^+Gr1^+$ cells, mixed with either $1x10^5$ LLCpc or LLC-CIITA cells (ratio 10:1). Control groups were injected with the same number of LLCpc or LLC-CIITA cells alone. Tumor size was measured weekly by caliper and sizes recorded as the tumor area (mm^2).

5. RESULTS

5.1 In vitro characterization of parental and CIITA-transfected LLC cells

As preliminary study to understand the biological properties of the tumor cell line analyzed in this investigation, we first determined the duplication time *in vitro* of LLC cells. This was found to be of 22 hours (***Fig.9***).

Subsequently, we assessed by flow cytometry the MHC class I and class II cell surface phenotype of parental LLC cells. MHC class I molecules were expressed (***Fig.10A***), whereas MHC class II molecules were not expressed (***Fig.10C***). Lack of expression of MHC-II molecule could not be rescued even after treatment with 300 U/ml of IFN-γ (***Fig.10D***), a cytokine which strongly increase MHC-II expression by acting on the upregulation of transcription of CIITA-encoding AIR-1 gene (***Fig.8***). IFN-γ is known to upregulate also MHC class I expression (*Yang I.et al.,2004).* In this case the IFN-γ treatment indeed increased MHC-I expression (***Fig.10B***).

Based on the above results, we investigated whether stable transfection of exogenous CIITA may induce MHC class II gene expression. CIITA cDNA was inserted into a vector containing a gene conferring resistance to neomycin (G418). Thus we first assessed the minimal dose of G418 capable to kill all parental LLC cells. The concentration of 1mg/ml was sufficient to kill 100% of the cells in 5-7 days. Stable CIITA plasmid and control plasmid transfectants were established by Lipofectamine in presence of the above concentration of G418 (***Fig.11B*** *and* ***11C***). Exogenous CIITA was able to induce expression of MHC class II genes and corresponding molecules indicating that parental LLC

cells do not have an intrinsic defect in the transcription of these genes, provided CIITA is expressed (***Fig.11C***). CIITA-transfectants expressing MHC-II molecules were further enriched by sorting, followed by cloning in limiting dilution conditions. LLC-CIITA clone E10 was chosen for further studies (***Fig.11D***).

5.2 In vivo studies of parental and CIITA-transfected LLC cells in C57Bl/6 mice

LLC cells transfected with CIITA display a reduced tumorigenicity *in vivo*. In fact, mice injected with LLC-CIITA cells were tumor-free for longer time than mice injected with parental tumor cells. After two weeks from LLCpc injection 100% of mice developed palpable tumors while only 60% developed tumors after injection of LLC-CIITA. Importantly, 40% of LLC-CIITA injected mice remained tumor-free after the 28 day observation period (***Fig.12A***). Of relevance, the growth kinetics and the size of CIITA-expressing tumors were significantly reduced compared to parental tumors. In fact, after 14 and 21 days from LLCpc cells injection, mice had an average tumor size double than mice injected with LLC-CIITA. 28 days after injection, the average size of LLCpc tumors was three times higher than LLC-CIITA tumors (***Fig.12B***). The fact that LLC-CIITA cells had a reduced growth but were not completely rejected by the syngeneic host, as it was found for other tumor models *(Meazza R. et al. 2003; Frangione V. et al. 2010)* suggested that the immune response against CIITA-tumors was not entirely protective. In order to investigate this aspect in more detail, we analyzed the tumor tissues originated from LLCpc or LLC-CIITA injection. As expected *ex vivo* parental LLC tumor cells were MHC-II negative (***Fig.13D***) ; instead, *ex vivo* LLC-CIITA tumor cells although still positive for

MHC class II, displayed a reduced expression with respect to the original injected cells (compare histograms in ***Fig.13A*** and ***13C***). This may partially explain why LLC-CIITA, although immunogenic, were not fully rejected *in vivo.*

5.3 Analysis of the protective potential of spleen cells and T cell subpopulations from CIITA-injected mice by Adoptive Cell Transfer

In order to evaluate the ability of splenocytes from CIITA-tumor vaccinated mice to confer protection from tumor growth, adoptive cell transfer experiment were set up. C57BL/6 naïve female and male mice were s.c. coinjected with $5x10^4$ LLC parental tumor cells mixed with either total splenocytes, purified $CD4^+$ or $CD8^+$ cells from immunized mice (still tumor free at 28 days post-CIITA-tumor injection) or from naïve control mice. All mice coinjected with LLC parental tumor cell and total splenocytes from immunized mice were tumor free up to 28 days. Moreover 40% and 20% of mice coinjected with LLC cells mixed with $CD4^+$ or $CD8^+$ cells from immunized mouse, respectively, were tumor free at the same time. Importantly, 20% of mice coinjected with LLC cells mixed with $CD4^+$ from immunized mice never developed tumor. Instead, all mice of the corresponding control groups developed tumors 21 days after injection (***Fig.14A*** and **14C**). Interestingly, mice injected with LLC cells mixed with total splenocytes, purified $CD4^+$ or $CD8^+$ cells from immunized mice showed an average tumore size almost three times smaller than corresponding control mice (***Fig.14B*** and ***14D***).

Taken together these results indicate tha a)- immune spleen cells from LLC-CIITA vaccinated mice can protect naïve mice from parental tumor take in adoptive cell transfer approaches; b)- both $CD4^+$ and $CD8^+$ T cells can act as effectors against the tumors if previously triggered by MHC-II positive tumor cells.

5.4 Animals injected with CIITA-tumors profoundly modify the tumor-related microenvironment

Tumor development and its growth are accompanied by a series of homeostatic modification in the host that often result in the generation of a pro-tumor microenvironment favouring tumor expansion and spread, while antagonizing anti-tumor immune responses. Among hemopoietic derived cells that are affected toward a pro-tumor growth function, an important role is played by so called myeloid-derived suppressor cells (MDSC), a population of $CD11b^+/Gr\text{-}1^+$ myeloid cells expanded dramatically during tumor progression. MDSC can inhibit the function of T cells and dendritic cells, contributing to tumor immune escape. We observed that LLCpc tumor-bearing mice had a dramatic splenomegaly, three to five times the volume of healthy mice. This splenomegaly was not observed or was only moderate in mice injected with CIITA-transfected tumors (***Fig.15*** *top panels*). Cell surface phenotype of the spleen cells showed that LLCpc tumor-bearing mice displayed a large population of $CD11b^+/Gr\text{-}1^+$ cells, accounting for 30-50% of the total spleen. In naïve mice, this population accounted for only 2-3% of spleen cells. Interestingly, LLC-CIITA injected mice, which were protected from tumor growth had the same proportion of $CD11b^+/Gr\text{-}1^+$ spleen cells as the naïve mice (***Fig.15*** *bottom panels*). Thus a large cell population with the phenotype of

MDSC is rapidly and massively accumulated in spleen cells of tumor-bearing mice but not in mice injected with LLC-CIITA tumor cells. These results suggest that CIITA-tumor cells can possibly affect both expansion and spleen accumulation of $CD11b^{+}/Gr\text{-}1^{+}$ contributing to tumor rejection. We further investigated whether these cells could bear functional importance in tumor development and/or in supppression of possible immune responses against the tumor. To investigate this aspect, two groups of C57BL/6 mice were coinjected with $CD11b^{+}/Gr1^{+}$ cells (derived from a spleen of LLCpc tumor-bearing mouse) mixed with LLCpc or LLC-CIITA tumor cells (ratio 10:1). All mice coinjected with LLCpc and $CD11b^{+}/Gr1^{+}$ cells developed palpable tumors (20-50 mm^{2}) within two-three weeks. The time of appearance of tumors was slightly accelerated with respect to the appearance of LLCpc tumors, although this result was not statistically significant. Of relevance, mice injected with either LLC-CIITA mixed with $CD11b^{+}/Gr1^{+}$ or with LLC-CIITA alone displayed virtually the same tumor appearance and tumor growth kinetics. At three weeks post-injection all mice coinjected with LLC-CIITA and $CD11b^{+}/Gr1^{+}$ cells were tumor free, and only 25 % of mice injected with LLC-CIITA cells alone developed tumor with an average size of 10 mm^{2}. Importanly, 35% and 50% of mice within these groups, respectively, did not develop tumors at all during the 5 week observational period. Therefore, mice injected with LLC-CIITA mixed with $CD11b^{+}/Gr1^{+}$ cells were equally protected from tumor growth as the ones injected with LLC-CIITA alone (***Fig.16A*** *and* ***16B***).

5.5 In vivo studies of parental and CIITA-transfected LLC cells in CD11c.DOG mice

A crucial point of our approach is to define whether CIITA-tumor cells may directly act *in vivo* as surrogate APC for their own tumor antigens in place of professional antigen presenting cells (APC) like dendritic cells (DC), and in so doing trigger and prime naïve tumor-specific CD4+ TH cells. In order to better investigate this point we made use of the transgenic CD11c.DOG mice which can be deprived of DC by conditional treatment with Diphteria Toxin (DT) (***Fig.19***). Indeed treatment with DT resulted in the drastic reduction of the CD11c-high/MHC-II+ DC subpopulation (***Fig.20***). Moreover, DT treatment in CD11c. DOG mice caused a splenic reduction of those proportionally low but existing B cells, $CD4^+$ and $CD8^+$ T cells which express high levels of CD11c surface molecules. Interestingly DT treatment resulted in an increase of neutrophils as compared to control spleens.

A protocol was then set up to analyze the behaviour of parental LLC or LLC-CIITA tumor cell growth in these mice. CD11c.DOG mice were daily treated with DT for eleven days. After two days of treatment the mice were injected with $2x10^5$ LLC-CIITA cells or LLCpc cells. DT-untreated mice were used as controls. Tumor size was measured using a caliper at weekly intervals. As shown in Figure 11, CD11c.DOG mice depleted of DC cells and injected with LLC-CIITA cells were tumor-free for a longer time than similarly treated mice injected with LLCpc. In fact, three weeks after immunization, these mice showed an average tumor size of 20 mm^2 (***Fig. 21F***) ; in contrast, control group mice injected with parental LLC cells showed an average tumor size six times greater (120 mm^2) (***Fig. 21B***).

Moreover, tumor growth in DT-treated mice injected with LLC-CIITA was even slower than the growth of LLC-CIITA tumors in DT-untreated mice (***Fig. 21 D***).

The above experiment was conducted several time with similar results (see ***Fig. 22***). The above results indicate that the depletion of dendritic cells in CD11c. DOG mice does not affect the growth retardation of LLC-CIITA tumor and thus give support to the idea that the important APC *in vivo* that prime tumor-specific TH cells are indeed the CIITA-transfected, MHC class II-positive tumor cells.

6. DISCUSSION

There are numerous evidences in favor of an immunological surveillance against tumors, in fact, several studies have reported tumor regression due to immune responses, at least in experimental animals *(reviewed in Dunn J.P. et al., 2004 ; Accolla R.S. et al., 2010)*, demonstrating that the immune system can be "educated" to selectively eliminate cancer cells. It is generally accepted that the *in vivo* adaptive anti-tumor immune responses are partially ineffective when CTL do not have sufficient support from TH cells that in order to be primed require the recognition of antigen presented by professional APC, like dendritic cells *(Steinman RM and Banchereau J., 2007)* The approach followed by our laboratory to obtain an optimal TH anti-tumor cells response is based on the concept that if tumor cells can optimally express MHC-II molecules, these cells may function as surrogate APC for their tumor antigens and thus trigger an effective immune response. To obtain these "optimally MHC-II expressing tumor cells", tumor cells were engineered with the cDNA coding for CIITA (MHC class II transactivator) the major transcriptional activator of MHC class II genes, discovered in our laboratory (*Accolla R.S. et al.,1986);).* We chose this strategy in order to maintain the natural processing and surface expression of MHC class II molecules which do not take place when the cells are transfected with isolated alpha-beta MHC class II genes alone. Moreover, the expression of invariant chain (li) and other accessory molecules like DM, necessary for the correct physiology of antigen presentation, are induced and/or optimized by CIITA. This approach has shown promising results in a series of tumor cell lines from mice of Balb/c (MHC H-2d) strain. CIITA transfected tumors could be rejected effectively, generating tumor specific T helper cell (TH) triggering, immunological memory and protection even against parental tumor *(Meazza et al.,2003; Mortara L. et al., 2006; Mortara L. et al., 2009; Frangione V. et al.,*

2010; see also Accolla RS and Tosi G, 2012; Accolla RS and Tosi G, 2013) Considering the genetic homogeneity of inbred strains and having in mind a possible application of this strategy to highly genetic diverse populations as humans, it was crucial to assess whether similar results could be extended to mouse strains with different background. The results presented in this thesis show that LLC tumor cells of the H-2b background could be rendered highly immunogenic and rejectable *in vivo* by CIITA-mediated gene transfer confirming the general applicability of our tumor vaccination model. In fact, the presence of CIITA-induced MHC-II molecules in tumor cells was instrumental to trigger antigen-specific antitumor response capable of protecting *in vivo* the vaccinated animals from tumor take, and, importantly, to protect them against the rechallenge with parental tumor cells. In LLC-CIITA vaccinated mice long-lasting tumor-specific CD4+ TH cells were generated that may protect naïve syngeneic C57BL/6 recipients from tumor take or significantly reduce tumor growth upon adoptive cell transfer. Although it was very suggestive from previous findings of our laboratory that tumor cells may act as APC *in vivo*, in the present study we provide the first evidence that CIITA-tumor cells act themselves as trigger of tumor specific TH cell priming *in vivo* and that this trigger does not require, or at least is not totally dependent from, dendritic cells the professional APC considered the major cell type responsible for TH cell priming. This evidence was obtained by taking advantage of a recently described transgenic mouse model, the H-2b CD11c.DOG mice, in which a conditional knock-out of DC can be obtained by treatment with diphtheria toxin (DT). Indeed in DT-treated transgenic CD11c.DOG mice, originally shown by (*Hochweller K.,2008)* to be transiently deprived of DC and confirmed here, LLC-CIITA tumor cells were strongly retarded in their growth, in some case fully rejected, with a pattern virtually superimposable to the one observed in DT-untreated, DC undepleted mice.

Thus tumor cells can act as APC for MHC-II-restricted TH cells in the absence of DC. Furthermore from an immunological point of view these results show that alternative mechanisms of antigen presentation for TH cell priming *in vivo* not only can occur but they can occur in milieus other than lymphoid tissues. In LLC-CIITA vaccinated mice, long-lasting tumor-specific CD4+ TH cells were generated that may protect naïve syngeneic C57BL/6 recipients from tumor take or significantly reduce tumor growth upon adoptive cell transfer. Vaccination of mice with LLC-CIITA tumor cells is instrumental also in reorienting the tumor microenvironment and the tumor-dependent extra-tumor microenvironment from a pro-tumor behavior to an anti-tumor behavior. This has been demonstrated by our previous work in the Balb/C system and further reinforced here by the unprecedented finding that myeloid-derived suppressor cells (MDSC) dramatically increased in LLCpc tumor-bearing mice virtually disappeared in CIITA-tumor vaccinated mice. Furthermore, MDSC did not exert their suppressive function *in vivo* on the onset of adaptive immunity triggered by CIITA-tumor cells. In conclusion, these results open new ways for alternative strategies of anti-tumor vaccination and anti-tumor therapy based on the optimal activation of anti-tumor CD4+ TH cells by CIITA-driven MHC-II-positive tumor cells.

7. FIGURES AND LEGENDES

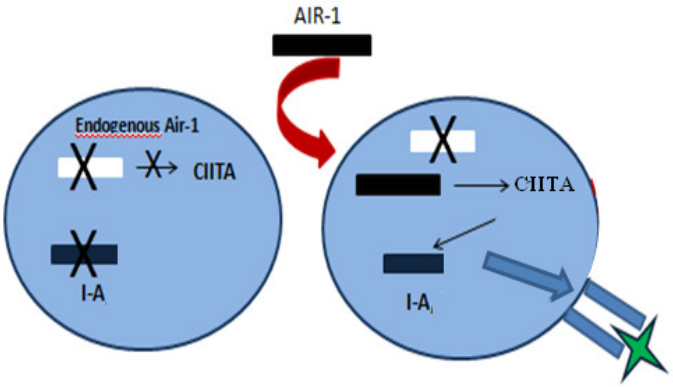


Fig.8

Expression of the AIR-1 locus-encoded CIITA induces MHC-II expression in LLC tumor cells.

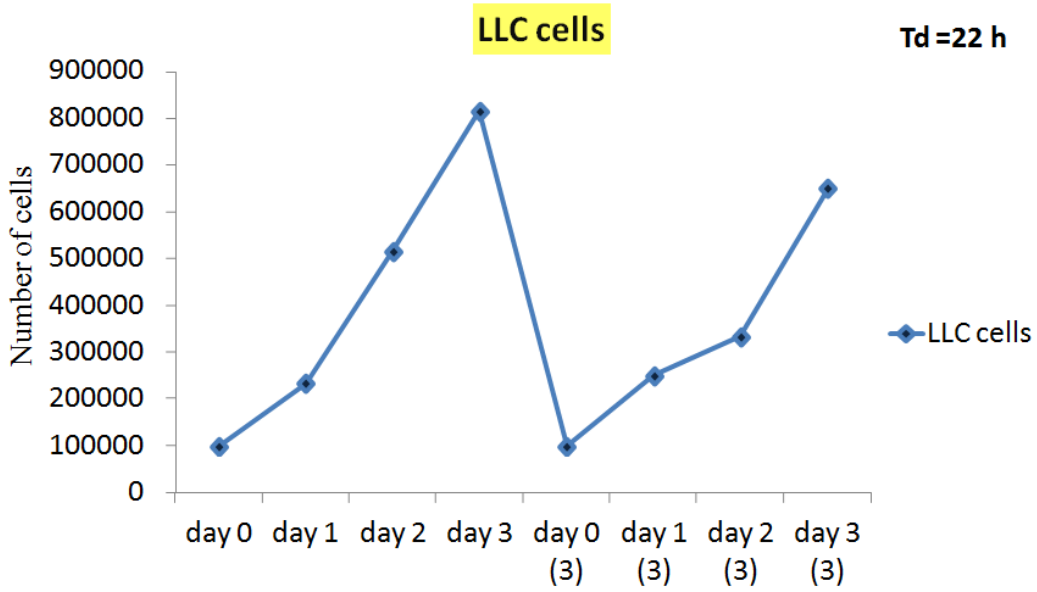


Fig.9 ***In vitro*** **growth curve of LLC cells.** Data are expressed in the ordinate as a number of LLC tumor cells at different time intervals (days in the abscissa). The duplication time of LLC cells was 22 hours.

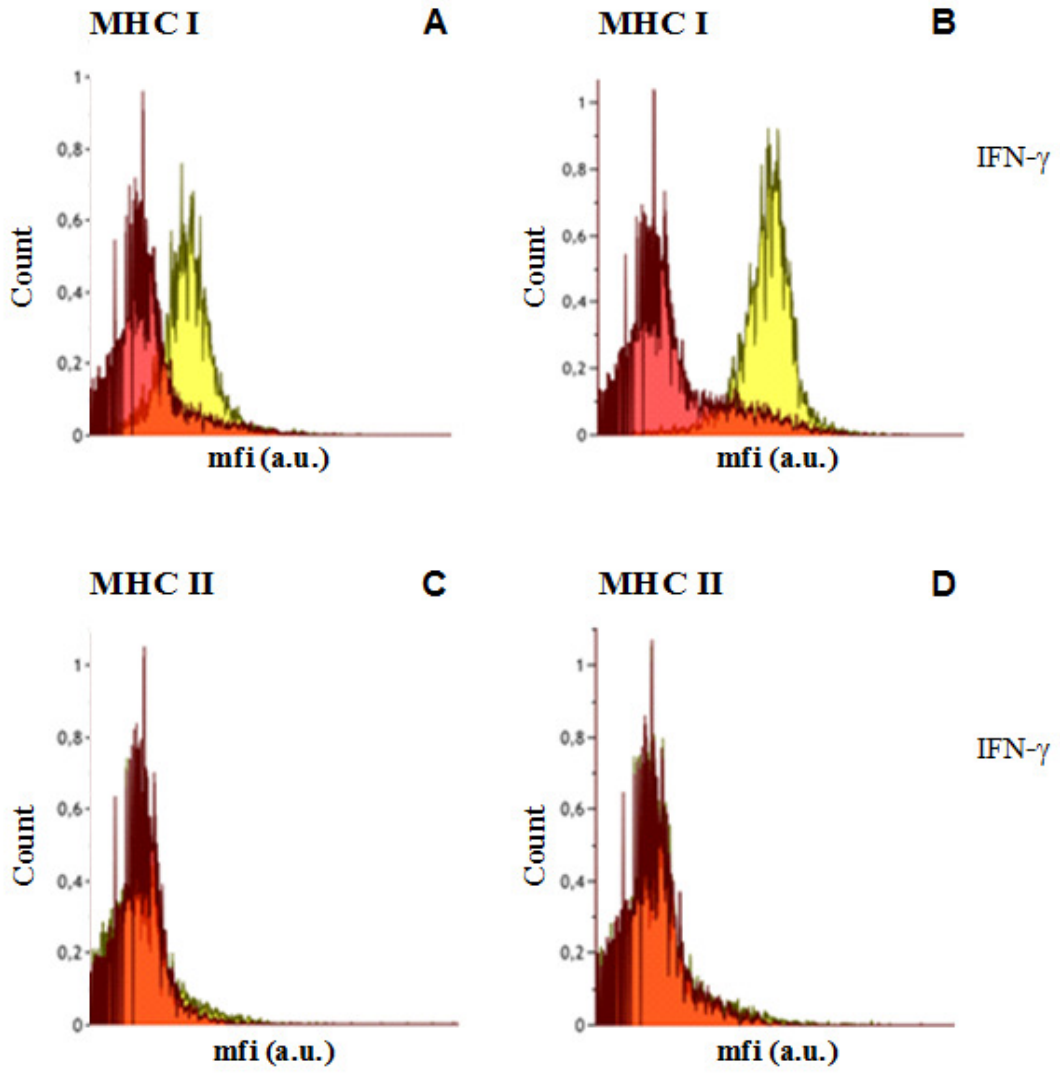


Fig. 10. Induction of MHC molecules in parental LLC cells by IFN-γ treatment. Parental LLC cells were treated with 300 U/ml of IFN-γ and tested for MHC-I and MHC-II molecules surface expression by FACS using appropriate antibodies.**A** and **C** depict the results of the expression of MHC-I and MHC-II molecules, respectively, in untreated parental LLC cells. **B** and **D** depict the results of the expression of MHC-I and MHC-II molecules, respectively, in parental LLC cells 96 hours after IFN-γ treatment.

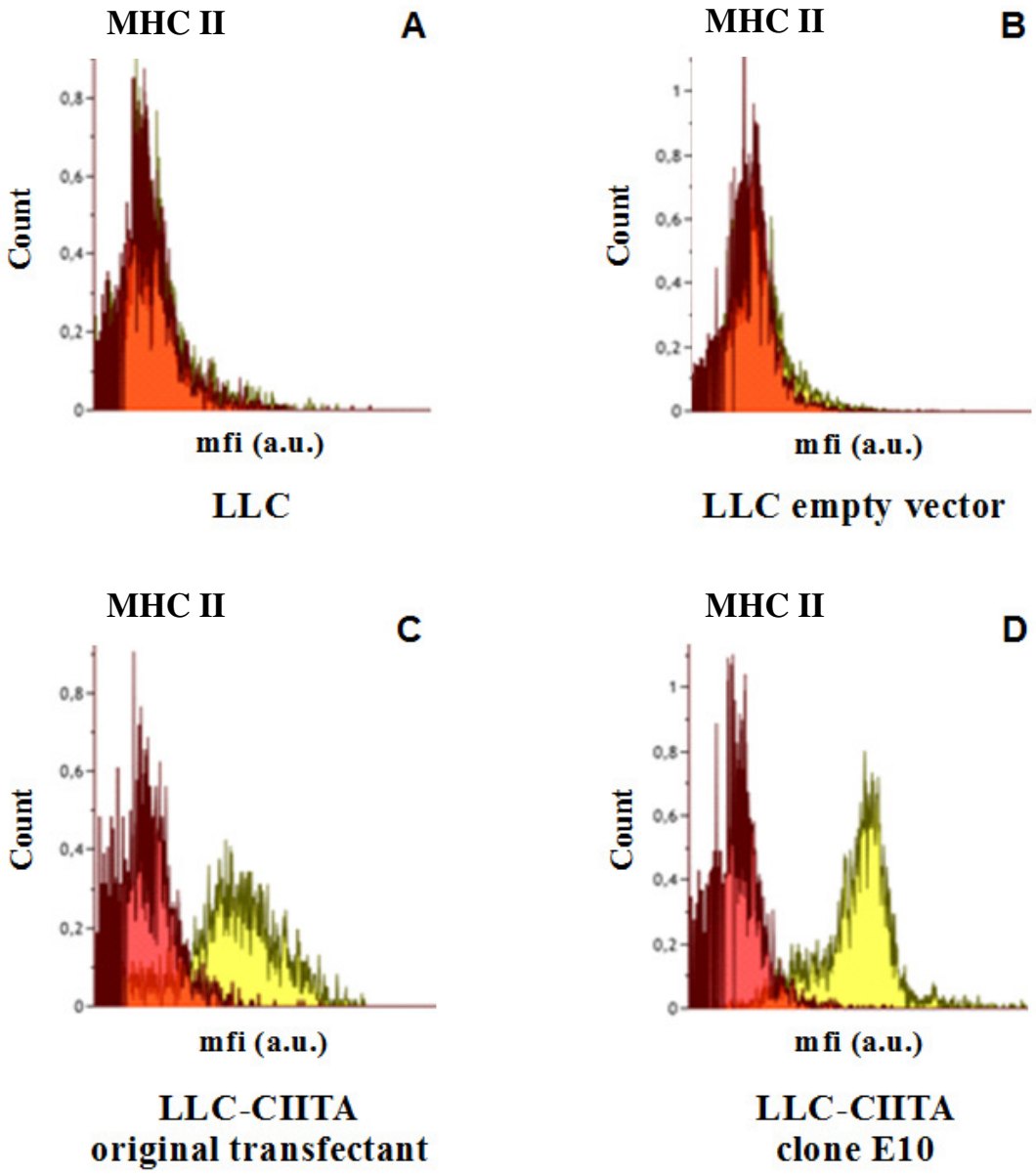
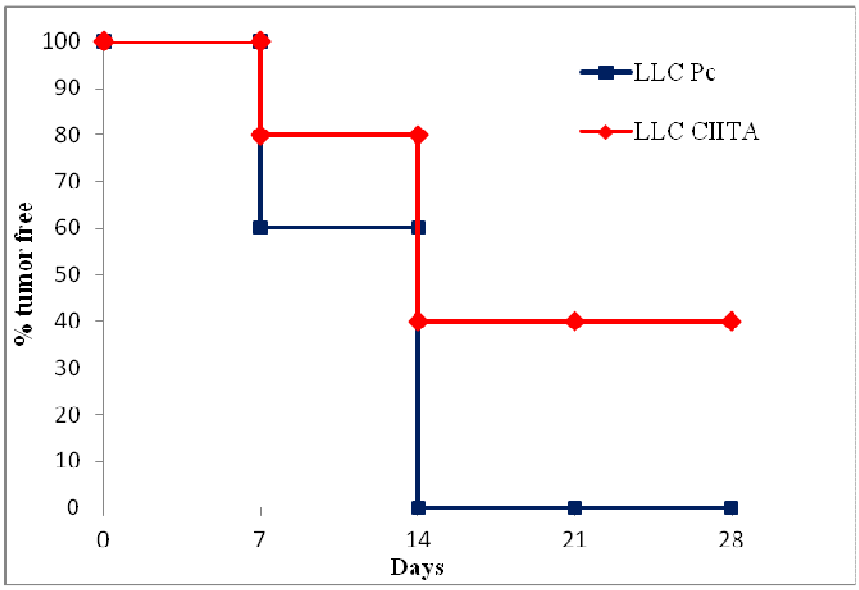


Fig. 11. MHC class II expression in LLC-CIITA trasfectants. Lewis Lung Carcinoma (LLC) cells were stably transfected with CIITA plasmid carrying the neomycin resistance gene (C and D) or with the empty vector LXIN as a control (B). Stable transfectants were selected in DMEM medium added with neomycin (1 mg/ml) and analyzed for the expression of CIITA-induced MHC-II cell surface molecules with a specific anti-MHC-II FITC-labelled mAb (yellow histograms).Original LLC-CIITA transfectant population(C); selected LLC-CIITA clone after cloning in limiting dilution conditions (D).

A



B

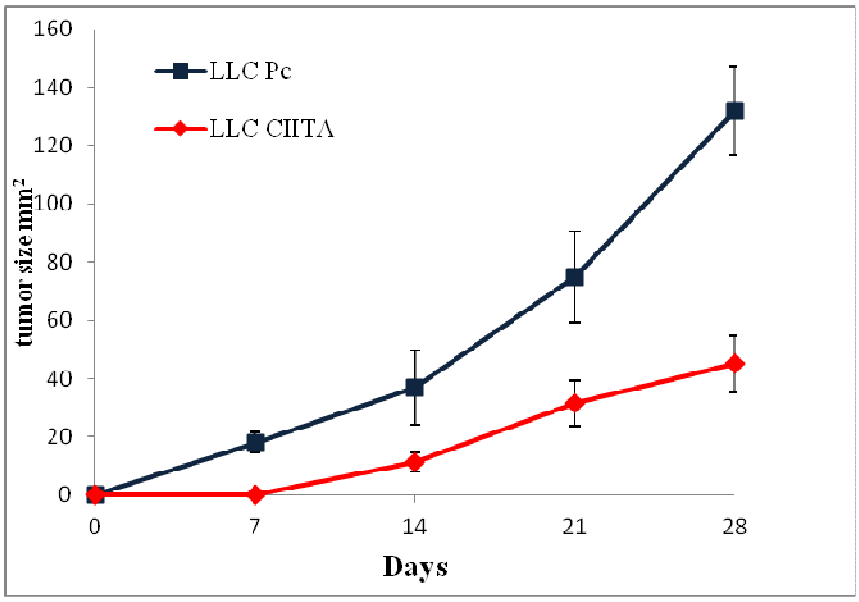


Fig. 12. **Mice injected with LLC-CIITA cells are tumor-free for a longer time than mice injected with parental tumor cells**. C57BL/6 mice were injected with either LLCpc or LLC-CIITA E10 clone cells (five mice per group). ***A*****,** percentage of tumor-free mice at different time intervals. One hundred percent of mice injected with LLC tumor cells developed palpable tumors within 2 weeks. Instead, 40% of mice injected with LLC-CIITA tumor cells were tumor free up to 4 weeks. ***B*****,** LLC-CIITA tumors showed an average tumore size significantly reduced compared to parental tumor.

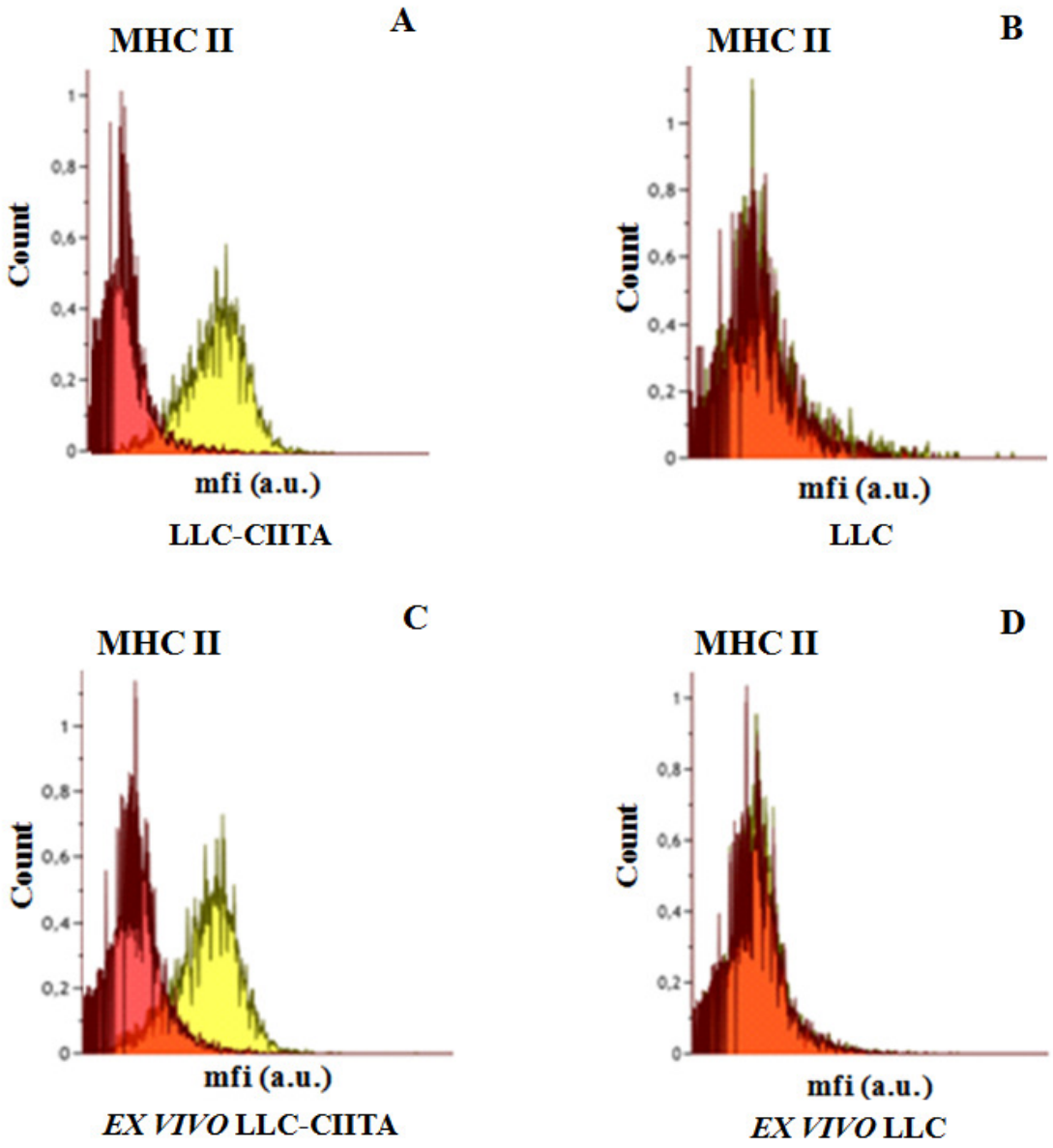


Fig. 13 *Ex vivo* tumors evaluation based on MHC-II molecules surface expression. LLC-CIITA cells before mouse injection **(A);** *ex vivo* LLC-CIITA tumor cells (**C).** Parental LLC cells before mouse injection **(B)**; *ex vivo* parental LLC tumor cells **(D).** *ex vivo* Parental LLC and LLC-CIITA tumors of similar size were excised, four weeks after tumor injection, from C57BL/6 mice and put in culture. After 2 weeks, cell cultures were phenotypically analyzed by immunofluorescence and FACS for the expression of MHC-II cell surface molecules with a specific anti-MHC-II FITC- labeled mAb (yellow histograms).

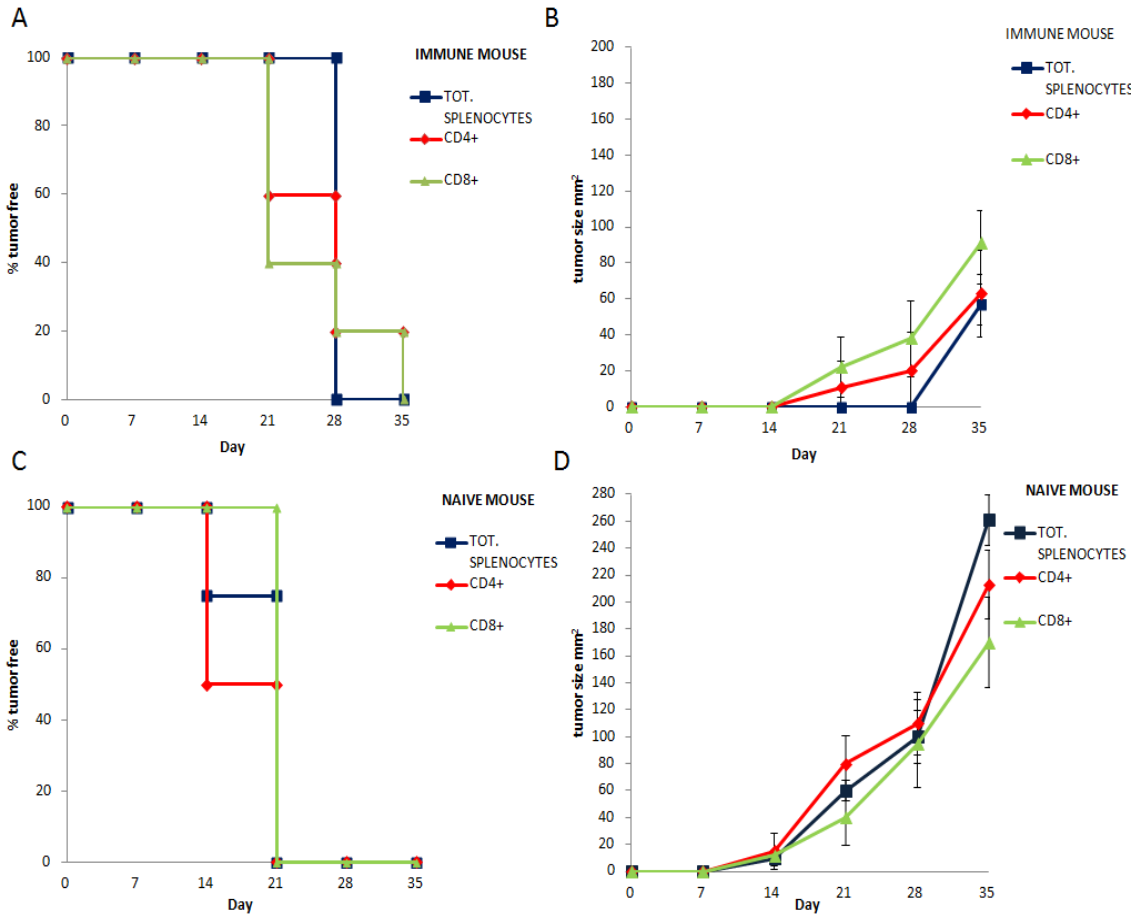


Fig. 14. Role of total splenocytes and T lymphocyte subpopulation in anti-tumor immunity after CIITA-vaccine.

Groups of five female C57BL/6 mice (5 to 8 weeks-old) were s.c. coinjected with $5x10^4$ LLC parental tumor cells mixed with total splenocytes, purified $CD4^+$ or $CD8^+$ cells from immunized mice or from a naïve mice (as a control). ***(A, C)*** Percentage of tumor-free mice at different time intervals. All mice coinjected with LLC parental tumor cells mixed with total splenocytes from immunized mice were tumor-free up to 28 days. 40% and 20% of mice coinjected with LLC cells mixed with $CD4^+$ or $CD8^+$ cells from immunized mice, respectively, were tumor free up to 28 days. Moreover, 20% of mice coinjected with LLC cells mixed with $CD4^+$ from immunized mice never developed tumor. All mice of the corresponding control groups developed tumor 21 days after injection. ***(B, D)*** Mice injected with LLC cells mixed with total splenocytes, purified $CD4^+$ or $CD8^+$ cells from immunized mice showed an average tumore size almost three times less than corresponding control mice groups.

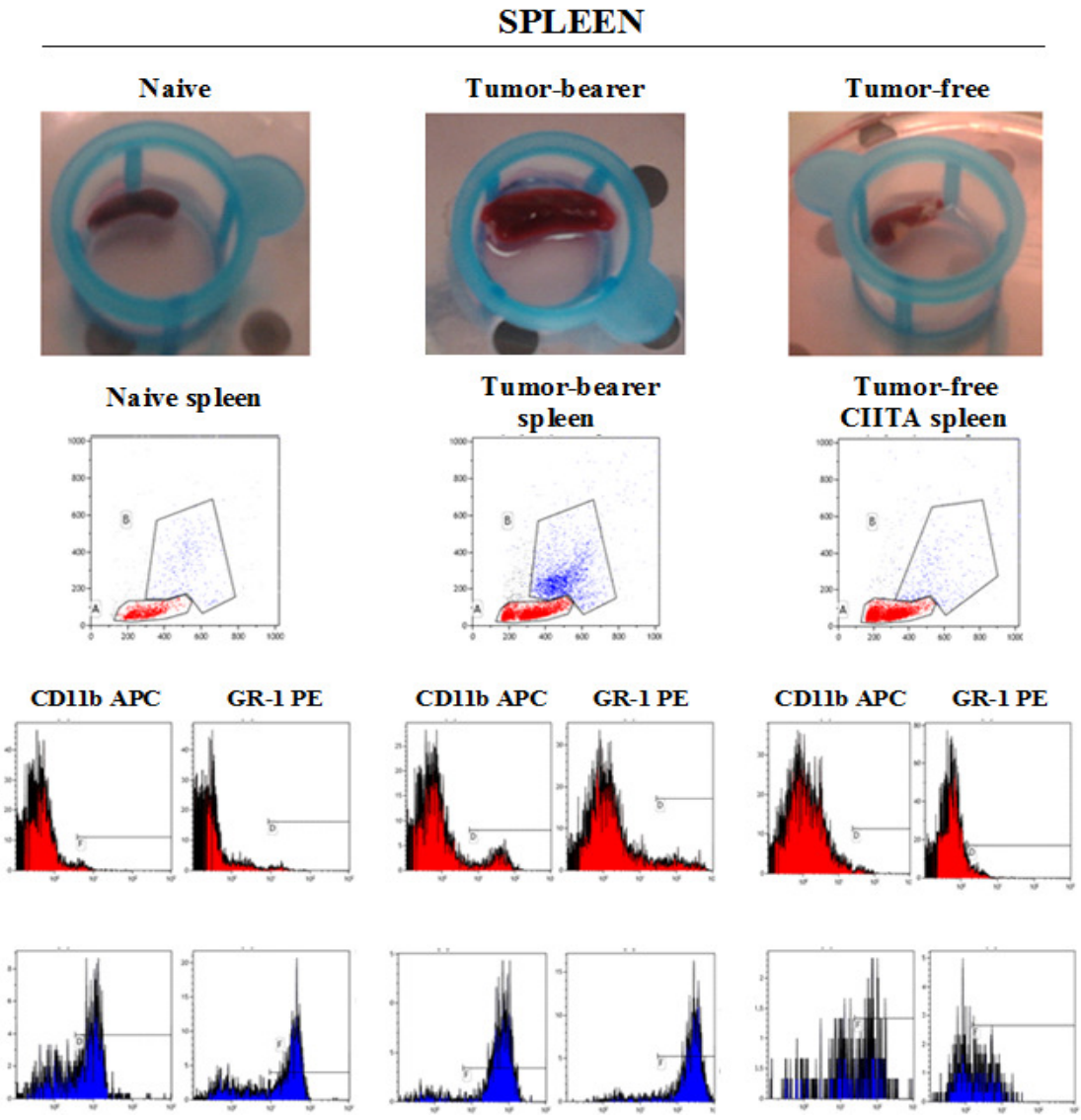


Fig. 15. Phenotypic analysis of CD11b$^+$ and Gr1$^+$ cells in spleens from different origin.

Upper panels: Photo of spleen harvested from LLC tumor-bearing mouse show an evident splenomegaly. The size is four times greater than spleens from naïve and LLC-CIITA tumor free mice. Lower panels: Citofluorometric analysis. Spleen cells from naïve, tumor-bearing and tumor -free C57BL/6 mice were isolated, as described in Materials and Methods, and stained using anti-mouse Gr-1 and anti-mouse/human CD11b antibodies. Spleen from LLC tumor-bearing mouse show a dramatic accumulation of CD11b$^+$ and Gr1$^+$ cells (blue gate), of very low proportion in naïve and LLC- CIITA tumor -free spleens.

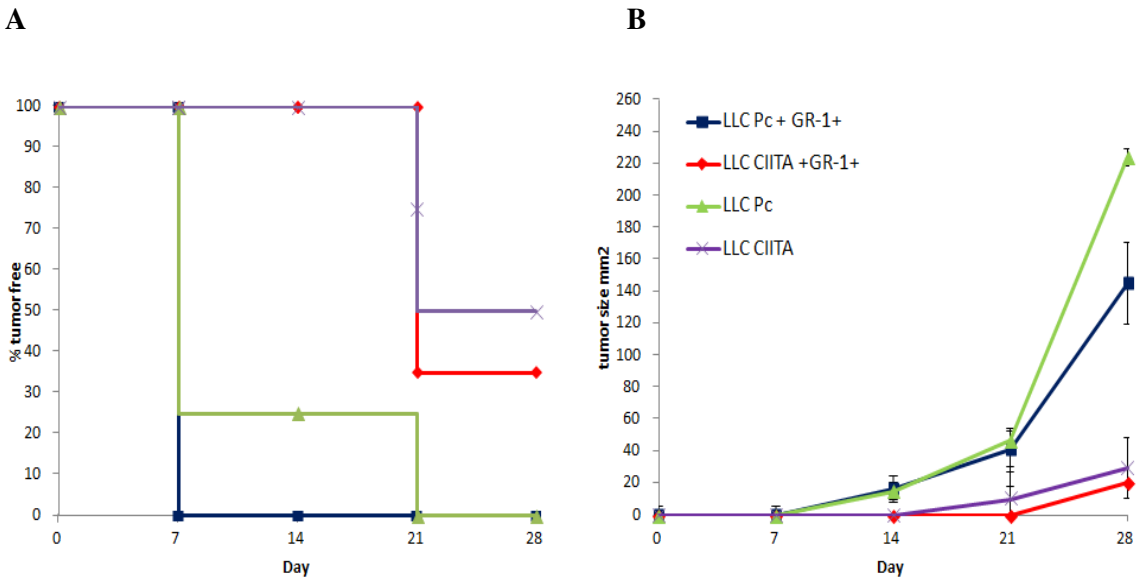


Fig. 16. Evaluation of *in vivo* effects of CD11b^{+}Gr1^{+} cells when coinjected with LLC or LLC -CIITA tumor cells.

Two groups of C57Bl/6 mice were coinjected with 1x10 ^{6} CD11b^{+}Gr1^{+} cells and 1x10 ^{5} LLC or LLC-Lxin CIITA E10-A12 clone cells (ratio 10:1); control mice groups were injected only with1x10 ^{5} LLC or LLC-Lxin CIITA cells.(***A***) Percentage of tumor-free mice at different time intervals. All mice coinjected with LLC cells and CD11b^{+}Gr1^{+} cells developed tumor up to two weeks and only 25% of corresponding control mice group were tumor free at the same time, but they also developed tumor after three weeks. Whereas, 100% of mice both injected with LLC-CIITA mixed with CD11b^{+}Gr1^{+}cells or with LLC-CIITA alone were tumor free after two weeks. Importantly , 35% and 50% of mice of these groups never developed tumor, respectively.(***B***) Mice injected with LLC-CIITA mixed with CD11b^{+}Gr1^{+}cells or with LLC-CIITA alone show an average of tumor size seven to twelve times less than corresponding control mice groups.

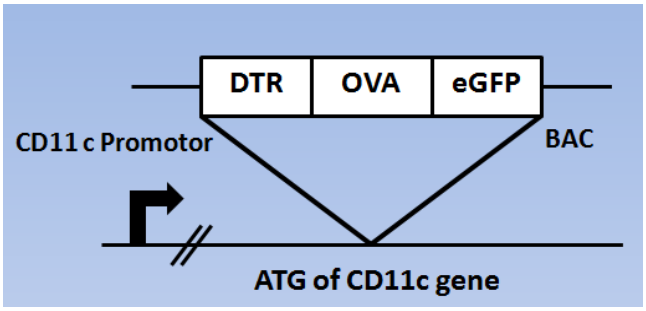


Fig. 17 Scheme of construct used to generate CD11c.DOG mice.
A fusion construct consisting of the human DTR, an ovalbumin 140–386 fragment, and eGFP was cloned into a BAC under the CD11c promoter by homologous recombination.

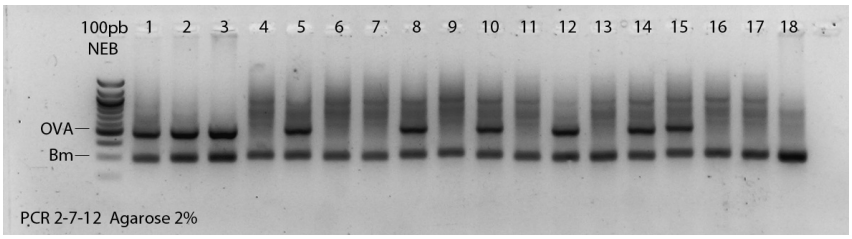


Fig. 18 CD11c.DOG mice genotyping
PCR samples were loaded on 2% agarose gel and DNA was visualized under UV exposure. Lines: 1,2,3,5,8,10,12,14 and 15 correspond to heterozygous CD11c.DOG mice. β microglobulin was used as housekeeping gene

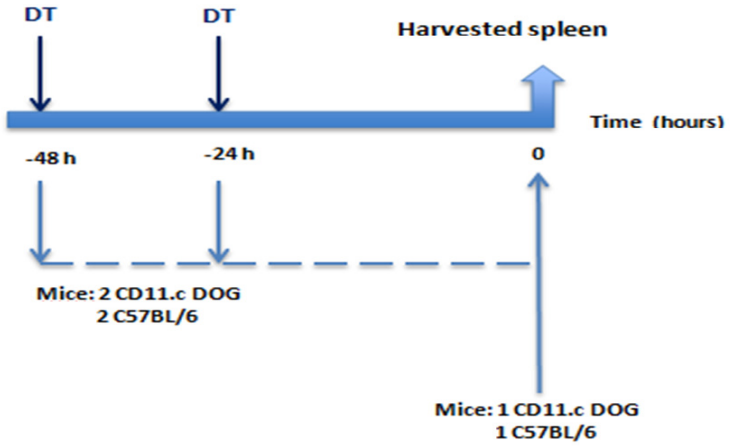


Fig.19 Experimental Scheme of DT administration in CD11c.DOG mice

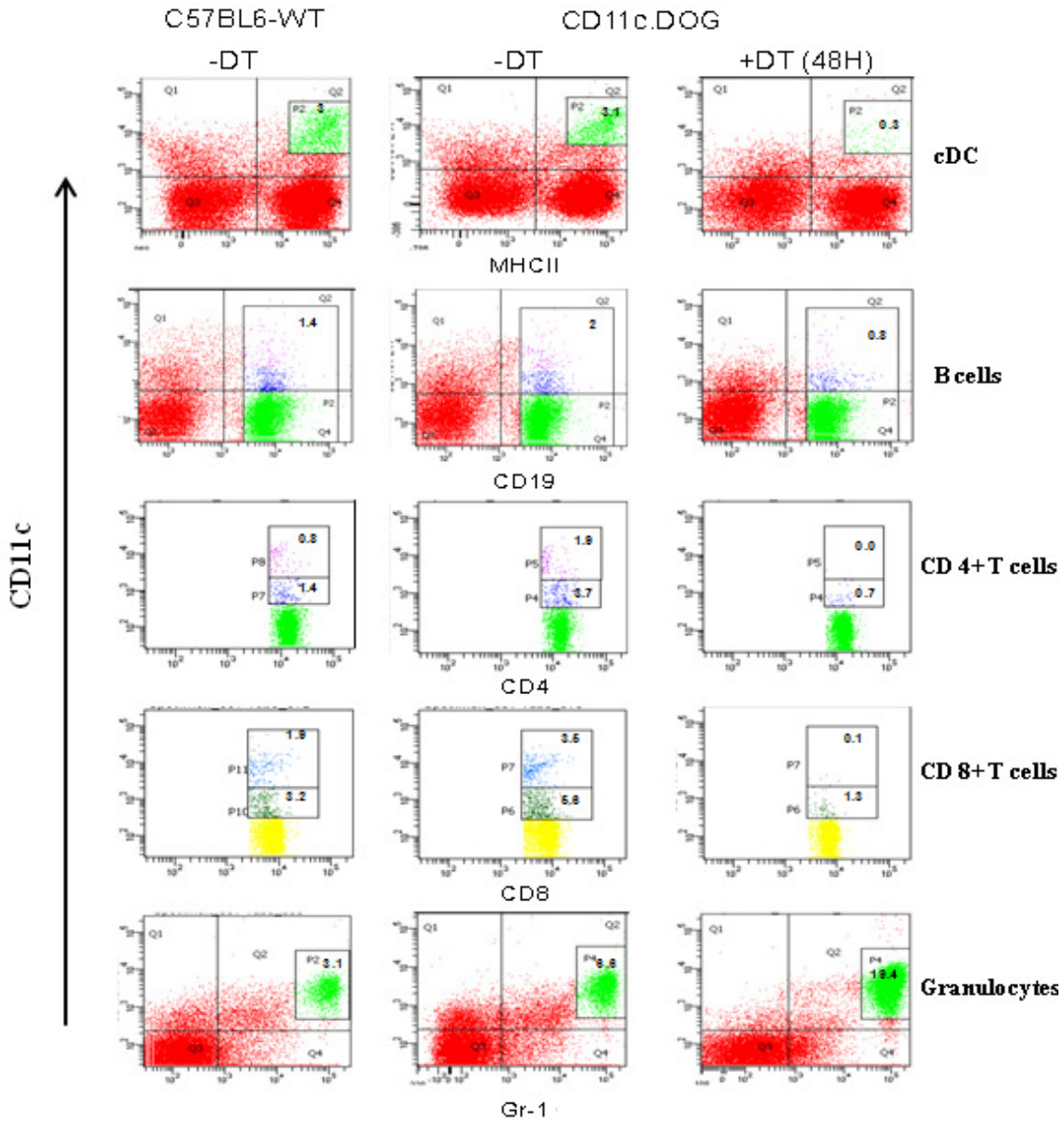


Fig. 20. Depletion of DC, B and T cells in CD11c.DOG mice after DT treatment. FACS dot blots of live splenocytes from untreated control mice (DT,left) or mice treated with DT (+DT, right). CD11c expression and depletion or increase of the following cell types 48 hrs after DT administration is shown (from top to bottom): cDC (gated on live splenocytes), B cells (gated on CD19+ cells), CD4 T cells (gated on CD3+ CD4+ cells), CD8 T cells (gated on CD3+ CD8+ cells), granulocytes (gated on Gr-1+ cells) The cut-off point for CD11c expression was obtained for isotype-matched irrelevant antibodies. Groups of 2 mice were used.

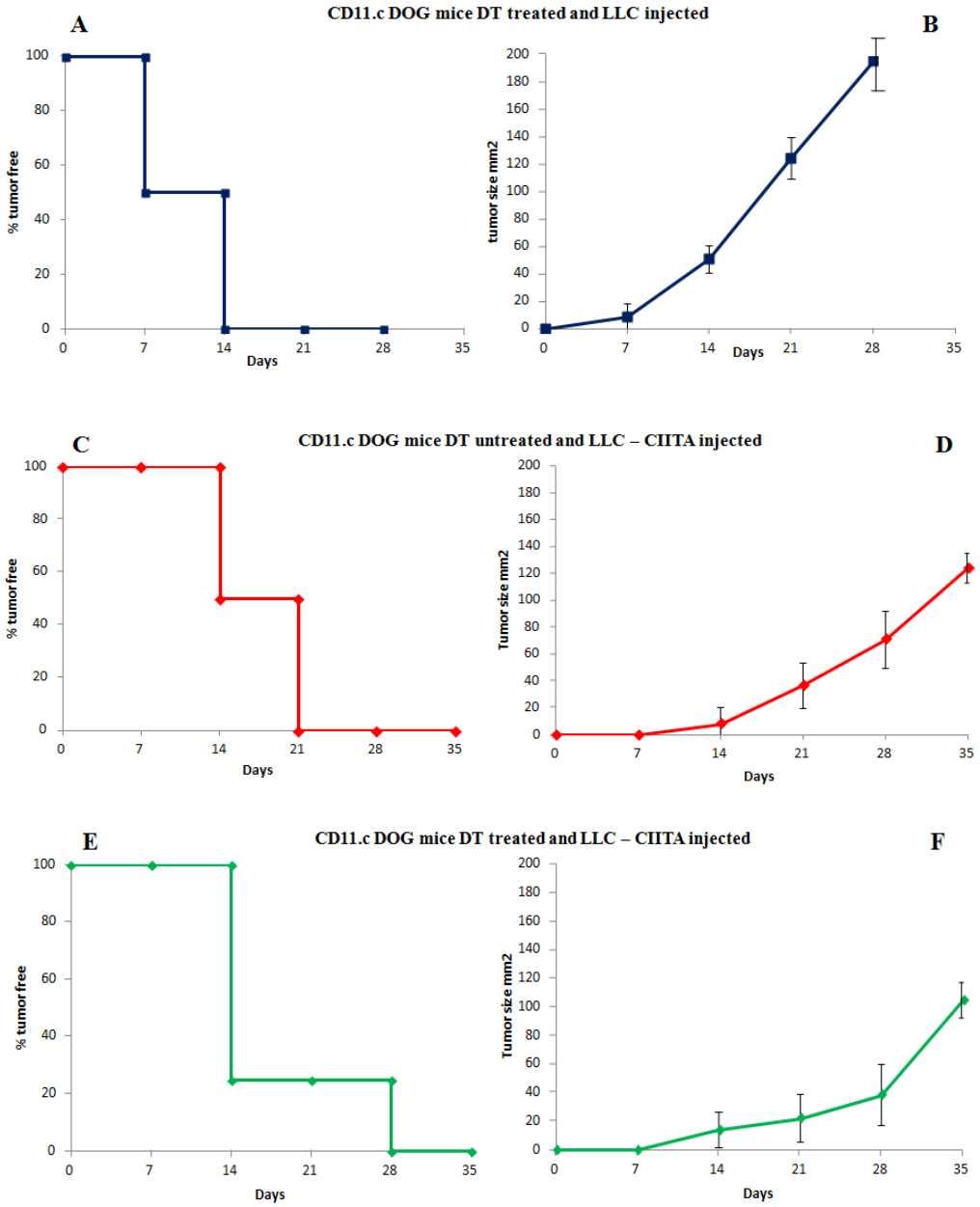


Fig. 21. Mice injected with LLC-CIITA cells DT treated and untreated show a strong reduction of tumor size compared to corresponding control mice groups injected with parental tumor cells. ***(A)*** Percentage of tumor-free DT treated mice at different time intervals. CD11c.DOG mice injected with 2x10 ^{5} parental LLC cells developed palpable tumors within 2 weeks. ***(C)*** 50% of CD11c.DOG mice DT untreated and injected with LLC-CIITA cells were tumor free for 3 weeks, instead, 25% of CD11c.DOG mice DT treated and injected with the LLC-CIITA cells were tumor free up to 4 weeks***(E)*** . ***(B)*** CD11c. DOG mice injected with parental LLC cells showed an average tumore size strongly increased than mice injected with LLC-CIITA cells. (***D, F***) CD11c.DOG mice untreated and injected with LLC-CIITA cells displaied an increase tumor growth compared to CD11c.DOG mice DT treated. Tumor size was measured using caliper at weekly intervals and was expressed as a multiple of the wider and smaller tumor diameters.

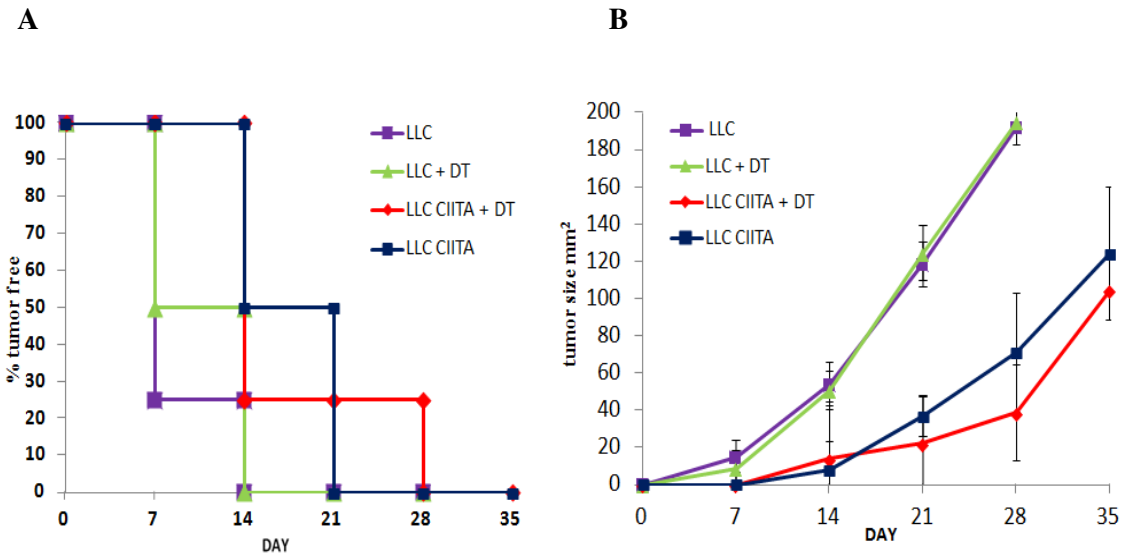


Fig. 22. Mice injected with LLC-CIITA cells DT treated and untreated show a strong reduction of tumor size compared to corresponding control mice groups injected with parental tumor cells.

CD11c.DOG mice DT treated or untreated (CTRL) were injected with 2x10 ^{5} LLC or LLC-Lxin CIITA E10-A12 clone cells (five mice per group). ***(A)*** Percentage of tumor-free mice at different time intervals. All CD11c.DOG mice DT treated and unteated injected with parental LLC cells developed palpable tumors within 2 weeks ; instead, at the same time 50% of CD11c.DOG mice DT untreated and injected with LLC-CIITA cells were tumor free and in the corresponding mice group DT treated, 25% of mice were tumor free up to 4 weeks. ***(B)*** CD11c.DOG mice DT treated and untreated , injected with parental LLC cells showed an average tumore size superimposable and strongly increased compared to corrisponding mice group injected with LLC-CIITA cells. Tumor size was measured using caliper at weekly intervals and was expressed as a multiple of the wider and smaller tumor diameters.

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6. **L. LOMBARDO,** G. FORLANI, A. TEDESCHI, R. S. ACCOLLA (2013)University of Insubria, Varese, Italy. Anti-tumor specific T-helper cell priming by CIITA-driven MHC-II-positive tumor cells in dendritic cell-depleted transgenic mice.15TH International Congress of Immunology, Milan, ITALY/ August 22-27 .

LETIZIA LOMBARDO: MEETINGS AND CONFERENCES

1. "Insubria Autumn School of Neuroimmunopharmacology (NIP)" . Varese- ITALY, Aula Magna di Via Dunant e sede di Villa Toeplitz. From 14 to 18 November 2011.

2. International Workshop on Viruses, Genes and Cancer . Palazzo Franchetti, Istituto Veneto di Scienze, Lettere ed Arti. October 25-27, 2012.

3. PhD Programme in Experimental Medicine and Oncology. The Immune Response against Tumors: novel Strategies of Immunoprevention and Immunotherapy. Aula Magna Pad. Bassani, Università dell' Insubria. 17-18 September, 2013.

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